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(54) Title: MILK PROTEIN HYDROLYSATE FOR ADDRESSING A BONE OR DENTAL DISORDER

(57) Abstract

A composition for prevention or treatment of a bone or dental disorder which comprises a milk protein hydrolysate, use of the milk protein hydrolysate in the manufacture of a composition for the treatment or prevention of a bone or dental disorder, and a method of treatment which comprises administering an effective amount of a milk protein hydrolysate. In preferred embodiments the milk protein hydrolysate is a hydrolysate of casein, in particular a caseinoglycomacropeptide (CGMP), a mimetic, homologue or fragment thereof in a bioavailable form which retains the ability of CGMP to inhibit bone resorption or bone loss; or favor calcium absorption, retention or calcification; or a combination thereof.

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Milk Protein Hydrolysate For Addressing A Bone Or Dental Disorder

The present invention relates to use of a milk protein hydrolysate in the manufacture of a composition for the treatment or prevention of a bone or dental disorder; and a method of treatment or prevention of a bone or dental disorder which comprises administering an effective amount of the milk protein hydrolysate.

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only".

Within the context of this specification, the term "bioavailable" means capable of crossing the intestinal barrier and being recovered in an active form in the blood. The term "bone or dental disorder" is taken to mean a condition in which calcium is implicated and affects the health of osseous tissue including calcium absorption, retention, resorption or calcification.

CGMP is used as an abbreviation for caseino-glycomacropeptide, CGMP-Ca and CGMP-Na are used as abbreviations for the calcium and sodium salts thereof. An alternative name for caseino-glycomacropeptide is k-caseinoglyco-peptide.

"Milk Protein Hydrolysate" is intended to mean a polypeptide which can be extracted from milk after at least partial hydrolysis eg with the enzyme trypsin.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined by peptide bonds or modified peptide bonds, i.e., peptide isosteres. They may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination or they may be cyclic, with or without branching. Cyclic, branched or branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide

derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., Post-translational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter, et al., "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990)182:626-646 and Rattan, et al., "Protein Synthesis: Post-translational Modifications and Aging", Ann NY Acad Sci (1992)663:48-62).

Within the context of this specification the terms "mimetic" and "homologue" are as defined in the art and taken to mean variants which retain the stated activity. "Variant" refers to a polypeptide that differs from a reference polypeptide, but retains essential properties. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by a genetic code. A variant of a polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Homology", as known in the art, is a relationship between two or more polypeptide sequences sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" or "homology" can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press,

New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine degree of identity or homology are codified in publicly available computer programs. Preferred computer program methods for determining identity or similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S. F., et al., J. Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following:
Algorithm: Needleman, et al., J. Mol Biol. 48: 443-453 (1970)
Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919 (1992)
Gap Penalty: 12 : Gap Length Penalty: 4

A useful program with these parameters is publicly available as the "gap" program from Genetics Computer Group. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

BLAST, which stands for Basic Local Alignment Search Tool (Altschul SF (1993) J Mol Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10), produces alignments of both nucleotide and amino acid sequences for determining sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologues. Other algorithms eg described in Smith R F and T F Smith (1992 Protein Engineering 5:35-51), can be used when dealing with primary sequence patterns and secondary structure gap penalties.

The BLAST approach (1993; Proc Nat Acad Sci 90:5873-7), incorporated herein by reference, searches matches between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. In this application,

threshold is set to at least 40% identity or homology.

CGMP is a milk protein hydrosylate. It is a sialynated macropeptide and accounts for approximately 40% of the κ-casein fraction. CGMP can be generated in several ways, including intestinal digestion of κ-casein where it is the first hydrolysis product resulting from the action of gastric proteases (chymosin and/or pepsin). CGMP is also released by chymosin action during the primary phase of milk clotting and goes into the whey fraction (sweet whey), while the N-terminal part of κ-casein co-precipitates with the casein fractions. CGMP can then be industrially separated from sweet whey and eluted in the form of sodium-CGMP (NaCGMP). Calcium-CGMP (CaCGMP) is generated from NaCGMP by a further step.

It is known that CGMP can adhere to various types of surfaces, e.g. polystyrene (J.R. Neeser et al., Infection and Immunity, **56**, 12, 3201-3208, 1988) and saliva-coated hydroxyapatite (J.R. Neeser et al., Oral Microbiol Immunol., **9**, 193-201, 1994), thereby preventing bacterial adherence to pellicle-coated teeth. It can thus protect against dental caries by inhibiting the adherence of cariogenic bacteria which induce caries. B. Chabance et al. (Biochimie **80**, 155-165, 1998) have shown that after eating, many peptides derived from α-, β- or κ-caseins, including CGMP, can be detected in stomach and blood for up to 8 hours and this indicates that CGMP can cross the intestinal barrier.

To date, the main biological action that has been attributed to the absorbed peptides is the reduction of platelet aggregation or blood clotting. The mechanism for this action has been shown to be due to the structural homologies that exist between fibrinogen and the N-terminal undecapeptide of CGMP (residues 106-116). It has been demonstrated in a series of *in vitro* and *in vivo* experiments that this undecapeptide can bind to integrin receptors on activated platelets, to which the γ-chain of fibrinogen normally binds and it thereby reduces clotting.

An example of a bone disorder which is recognised as a major public health problem is osteoporosis. Indeed, post-menopausal osteoporotic fractures affect about 1.5 million people per annum. About 300,000 new cases of osteoporotic hip, 650,000 vertebral and 200,000 distal forearm fractures are reported annually in the US. Mortality in the first year after hip fractures reaches 20% (Amer. J. Epidem. 1001-1005 (1993)) and the estimated cost of treatment of these patients is in the region $6-$10 billion annually.

While several effective drugs are available to reduce bone loss, there are also opportunities for nutritional alternatives which could be used in combination with or independently of drugs. Research to date on the nutritional prevention of bone loss has primarily focused on the more classical nutrients, such as calcium and vitamin D. There is a limit, however, to the ability of the classical nutrients to prevent bone loss.

Remarkably, it has now been found that a composition which comprises CGMP has a positive affect on maintenance of bone or dental health or treatment of bone or dental disorders. In *in vitro* tests, CGMP was also found to reduce the activity of the bone degradation cells (osteoclasts) and increase the activity of the bone forming cells (osteoblasts) indicating potential benefits of CGMP for bone health. In a growing animal model of bone loss CaCGMP was found to significantly reduce bone loss and there was greater retention of calcium in animals consuming either CaCGMP and NaCGMP. in vitro tests on bone forming and bone degradation cells (i.e. osteoblasts and osteoclasts). Furthermore it has been found that CaCGMP and NaCGMP can reduce bone loss in an animal model of menopausal bone loss (the ovariectomised rat). In addition, it has been found that different forms of CGMP and its peptides have benefits for bone health at different stages of the lifecycle. The effects of CGMP are unexpected because although the beneficial affect of drinking milk is well described it has now suprisingly been found that a milk protein hydrolysate has an effect on prevention or treatment of a bone or dental disorder.

Nevertheless, there is no indication in the prior art of the use of a milk protein hydrolysate, in particular CGMP, for prevention or treatment of a bone disorder eg by inhibiting bone resorption or bone loss; or favoring calcium absorption, retention or calcification; or a combination thereof in a mammal.

The present invention addresses the problems set out above.

Accordingly, in a first aspect the present invention provides a composition for prevention or treatment of a bone or dental disorder which comprises a milk protein hydrolysate.

In a second aspect the invention provides use of a milk protein hydrolysate in the manufacture of a composition for the treatment or prevention of a bone or dental disorder.

In a third aspect the invention provides a method of treatment of a bone or dental disorder which comprises administering an effective amount of a milk protein hydrolysate.

An advantage of the present invention is that it provides a composition which can be administered orally. This is both safer and more convenient for a patient than a conventional treatment administered by injection.

Preferably milk protein hydrolysate is capable of inhibiting bone resorption or bone loss; or favoring calcium absorption, retention or calcification; or a combination thereof.

Preferably the milk protein hydrolysate comprises a hydrolysate of casein , more preferably a caseinoglycomacropeptide (CGMP), a mimetic, homologue or fragment thereof in a bioavailable form which retains the ability of CGMP to inhibit bone resorption or bone loss; or favor calcium absorption, retention or calcification; or a combination thereof. More preferably it is a sodium or calcium salt of CGMP or a mixture thereof. Most preferably it is the sodium salt of CGMP.

Preferably the amount of CGMP in an embodiment of the composition is about 0.01% to about 10% by weight of dry matter, and preferably about 0.5% to about 5% by weight of dry matter.

Preferably an embodiment of the composition comprises calcium. Preferably a composition according to an embodiment of the invention comprises about 15% to about 64% the RDA of calcium per 250g (RDA calcium is 1000mg); ie about 75mg to about 320mg per 125g of the composition. More preferably it comprises about 50% of RDA calcium per 250g; ie 250mg per 125g of the composition.

Preferably an embodiment of the composition comprises a source of protein and at least 0.01% by weight based on dry matter of CGMP in a bioavailable form. As a source of protein, a dietary protein is preferably used in a suitable form. Milk protein, whey protein, soy protein or a mixture of two of more thereof are particularly preferred. The composition may also contain a source of carbohydrate and/or a source of fat.

Preferably the invention provides a food supplement and/or a food product which contains at least 0.01% by weight based on dry matter of CGMP in a bioavailable form,

for inhibiting bone resorption or bone loss; or favoring calcium absorption, retention or calcification; or a combination thereof in a mammal.

Preferably, the composition comprises a source of carbohydrate, a source of fat or a source of protein or a combination comprising at least two thereof. More preferably, it comprises from about 15 to about 25% total calories protein, from about 10 to about 30% total calories fat, and from about 40 to about 60% total calories carbohydrate. Preferably, at least a portion of the protein is provided as caseinoglycomacropeptide (CGMP).

Preferably, the composition comprises sufficient minerals and vitamins to meet daily requirements.

Preferably the nutritional composition is incorporated into a food formula, for example an infant formula.

Preferably, the composition comprises from about 1 to about 50 grams, preferably from 5 to about 25 grams and most preferably from 5 to about 10 grams CGMP per 100 g of food formula.

Preferably the composition is administered to provide sufficient CGMP to inhibit bone resorption or bone loss; or favor calcium absorption, retention or calcification; or a combination thereof in humans or companion animals. The exact amount could be determined without difficulty by administering CGMP until the correct effect is seen. The dose of CGMP is preferably from about 1 to about 50 grams per day, more preferably from 9 to about 18 grams per day and most preferably from 3 to about 6 grams per day consumed at two or three times throughout a day.

Preferably CGMP is obtained by an ion-exchange treatment of a liquid lactic raw material containing CGMP. Suitable starting materials of lactic origin may include for example:

- the product of the hydrolysis with rennet of a native casein obtained by acidic precipitation of skimmed milk with a mineral acid or acidifying ferments, optionally with addition of calcium ions,
- the hydrolysis product of a caseinate with rennet,
- a sweet whey obtained after separation of casein coagulated with rennet,
- a sweet whey or such a whey demineralized, for example, by electrodialysis and/or ion exchange and/or reverse osmosis,

- a concentrate of sweet whey,
- a concentrate of whey proteins obtained by ultrafiltration and diafiltration of sweet whey.
- mother liquors of the crystallization of lactose from a sweet whey,
- a permeate of ultrafiltration of a sweet whey.

A preferable method of obtaining CGMP is described, for example, in WO 98/53702 and includes the decationization of the liquid raw material, such that the pH has a value of 1 to 4.5, bringing the said liquid into contact with a weak anionic resin of hydrophobic matrix, predominantly in alkaline form up to a stabilized pH, then separation of the resin and the liquid product which is recovered, and desorption of CGMP from the resin.

Preferably a composition according to an embodiment of the invention comprises a probiotic bacterium. Preferably, the bacterium is a lactic acid bacterium. More preferably, it is selected from the group which comprises *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorous, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii; Lactobacillus paracasei, Lactobacillus reuteri, Lactobacillus helveticus, Lactobacillus brevis; Lactobacillus fermentum; Lactobacillus plantarum; Lactobacillus casei* particularly *L. casei, Lactobacillus paracasei* and *L. rhamnosus; Lactobacillus delbrueckii* particularly *L. delbrueckii* subsp *lactis, L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *bulgaricus;* and bifidobacteria particularly *Bifidobacterium infantis, Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium lactis Bifidobacterium longum;* and *Leuconostoc mesenteroides* particularly *L. mesenteroides* subsp *cremoris*. Most preferably the bacterium is *Lactobacillus acidophilus* La10 (ATCC 11975). An advantage provided by these bacteria is that they have a striking positive affect on enhancement of calcium absorption. Remarkably, the affects on bone formation and resorption are particularly striking when the composition comprises *Lactobacillus acidophilus* La10 (La10). Preferably a composition according to an embodiment of the invention comprises about 10^7 to about 10^9 cfu/ml or /g probiotic bacterium. More preferably it comprises about 10^8 cfu/ml probiotic bacterium.

Preferably a dose of about 250 to about 300g of a composition according to an embodiment of the invention is provided by two servings per day. More preferably the dose is about 250g per day ie two does of about 125g per day.

Preferably, an embodiment of a composition according to the invention comprises nutrients/non-nutrients selected from the group which comprises: between about 15 and about 50% of RDA per 250g of the composition of magnesium, calcium citrate malate or calcium citrate lactate or milk calcium, phosphorus, iron, iodine, zinc, copper, selenium; about 7.5 to about 25% RDA per 125g of the composition of vitamins D, D3, A, B1, B2, B6, B12, C, E, K, niacin, folic acid, pantothenic acid, between about 15 and about 33% of RDA per 125g of the composition of biotin; about 6 to about 40g per 250g prebiotics (eg fibres, cellulose); about 50 mg to about 2g per 250g of the composition of casein phosphopeptide (CPP); about 25 to about 50 mg per 250g of the composition of isoflavone; about 1.5 to about 2.5 ng per mg of protein of bioactive peptide derived from milk eg TGF- , PTHrp.

Additional features and advantages of the present invention are described in, and will be apparent from, the description of the presently preferred embodiments which are set out below with reference to the drawings in which:

Figure 1 shows mean [H^3] tetracycline excretion levels and standard error bars for control, NaCGMP and CaCGMP groups. Each line represents [H^3] tetracycline excretion (bone resorption) for each group over a 13 day period.

Figure 2 shows the effect of CaCGMP and NaCGMP on long-term loss of trabecualr bone density in the ovariectomised rat.

Remarkably it has now been found that different forms of CGMP (NaCGMP and CaCGMP) have biological effects on Ca absorption and bone metabolism. Without wishing to be bound by theory it is hypothesised that CaCGMP (containing 2.3% Ca) might maintain Ca in a soluble form in the intestine in a similar manner to casein phosphopeptides and thus increase Ca bioavailability. Secondly in light of its anti-adhesion properties and affinity for hydroxyapatite, it is hypothesised that CGMP peptides might have a direct biological action on bone, perhaps by the impairment of osteoclast adhesion to the bone surface and thereby reducing bone loss. In support of this hypothesis, studies have shown that the Na form of CGMP reduces the bone resorbing activity of isolating osteoclasts on dentine slices.

In order to test the hypothesises, the effects compositions comprising two forms of CGMP (NaCGMP and CaCGMP) were studied , at approximately 4.5% of the total diet, on bone loss in the tetracycline labeled rat, which is a validated animal model of bone

loss (Am. J. Physiol 259: R679-R689). Under circumstances of "induced" bone loss, this model has been shown to be highly sensitive to pharmacological and non-pharmacological inhibitors of bone loss (e.g. bisphosphonates, vegetable derived components) within a short space of time (<14 d). Additionally, due to the feasibility of daily collection of urine and feces during the experimental period, it was possible to use this model to measure the amount of Ca that was absorbed, retained and excreted (i.e. Ca balance). In summary, this animal model of bone loss was considered to be sufficiently versatile and sensitive for testing the short-term effects of diet-derived CGMP on bone loss and Ca absorption.

Preferably an embodiment of the composition comprises at least a source of protein and CGMP. Dietary protein is preferably used as a source of protein. The dietary proteins may be any suitable dietary protein; for example animal protein (such as milk protein, meat protein or egg protein); vegetable protein (such as soy protein, wheat protein, rice protein, or pea protein); a mixture of free amino acids; or a combination thereof. Milk protein such as casein, whey protein or soy protein are particularly preferred.

The composition may also contain a source of carbohydrate and/or a source of fat.

A preferred embodiment of the composition comprises a fat source, the fat source preferably provides about 5% to about 55% of the energy of the nutritional formula; for example about 20% to about 50% of the energy. The lipids making up the fat source may be any suitable fat or fat mixture. Vegetable fat is particularly suitable; for example soy oil, palm oil, coconut oil, safflower oil, sunflower oil, corn oil, canola oil, lecithins, or the like or a mixture of two or more thereof. Animal fat such as milk fat may also be added if desired.

A preferred embodiment of the composition comprises a source of carbohydrate. It preferably provides about 40% to about 80% of the energy of the nutritional composition. Any suitable carbohydrate may be used, for example sucrose, lactose, glucose, fructose, corn syrup solids, and maltodextrins, or a mixture of two or more thereof.

A preferred embodiment of the composition comprises dietary fibre. If used, it preferably comprises up to about 5% of the energy of the nutritional formula. The dietary fibre may be from any suitable origin, including for example soy, pea, oat, pectin, guar gum, gum arabic, fructooligosaccharide or a mixture of two or more thereof.

A preferred embodiment of the composition comprises one or more suitable vitamins and/or minerals may be included in an embodiment of the composition in an amount to meet the appropriate guidelines.

A preferred embodiment of the composition comprises one or more food grade emulsifiers may be incorporated into the nutritional formula if desired; for example diacetyl tartaric acid esters of mono- and di- glycerides, lecithin and mono- and di-glycerides or a mixture of two or more thereof. Similarly suitable salts and stabilisers may be included.

A preferred embodiment of the composition is enterally administerable; for example in the form of a powder, a liquid concentrate, or a ready-to-drink beverage. If it is desired to produce a powdered version of a nutritional formula, the homogenized mixture is transferred to a suitable drying apparatus such as a spray drier or freeze drier and converted to powder.

In a further embodiment, a typical food product may be enriched with CGMP. For example, a fermented milk, a yogurt, a fresh cheese, a renneted milk, a confectionery bar, breakfast cereal flakes or bars, drinks, milk powders, soy-based products, non-milk fermented products or nutritional supplements for clinical nutrition. Then, the amount of CGMP added is preferably of at least about 0.01% by weight.

In an alternative embodiment the composition may be incorporated in an article of confectionery, for example a sweet, or sweetened beverage.

The following examples are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application. Percentages and parts are by weight unless otherwise indicated.

Example 1: Preparation of CGMP.

Bovine sweet whey was concentrated to 17% dry matter, demineralized by electrodialysis, freed of cations on a strong cationic resin column, freed of anions on a weak anionic resin column and spray-dried in a drying tower. Its composition is indicated below:

	%

Proteins (GMP included)	11.7
Lactose	81.7
Ash	1
Lipids	1
Water	balance for 100

The demineralized whey powder was solubilized in deionized water. After cation removal the solution has an initial pH of 3.8. In the preceding plant, 392 kg of this solution was treated at the temperature of 8°C, while stirring it in the reactor in the presence of 23 kg of weak anionic resin of hydrophobic matrix based on polystyrene (IMAC HP 661®, Rohm & Haas, regenerated in OH- form) for 4 h. Stabilization of the pH at 4.89 indicates the end of the reaction. The liquid was drawn off and the resin was recovered as above.

After concentration of the liquid to 45% dry matter by evaporation, the concentrate was spray-dried in a drying tower.

Analysis of the concentrate by HPLC showed that the reaction removed 89% of the starting CGMP. Moreover, the powder contained 9.1% of whey protein, which corresponded to a yield of 90% of the whey proteins.

To recover CGMP, the resin was washed successively with deionized water, with 30 l of an aqueous solution at 0.5% HCl and with 30 l of deionized water, and the CGMP was eluted twice with 40 l of aqueous solution at 2% Ca(OH)2. Rinsing is carried out with 30 l of deionized water. After combining the eluate and rinsing volumes, the combination was concentrated to a volume of 25 l by ultrafiltration with a membrane having a nominal cut-off of 3000 daltons. The retentate was freeze-dried and 900 g of CGMP were obtained, corresponding to a yield of 80% relative to the starting CGMP.

Example 2: Resorption Of Dentine By Disaggregated Osteoclasts Isolated From Rat Bone

Materials and methods

Osteoclasts were isolated from femura and tibia dissected from 1 to 2 days old rats. The bone were curretted with a scalpel in MEM containing Earle's salt with 15 mM NaHCO3 and 10% foetal bovine serum (FBS). The cell suspension was added on ivory slices and the cells were allowed to adhere for 40 min incubation time. After washing off non-

adhering cells, individual slices were placed into 48-well tissue culture dishes and incubated for 24 hours with medium containing 10% FBS and different concentrations of CGMP as prepared in example 1. The cells were fixed and stained for tartrate resistant acid phosphatase (TRAP), a specific marker for osteoclasts and subsequently counted. The cells were then removed by ultrasound. After washing and drying, the slices were gold-sputtered and the pits scored using a tangential light source. In one experiment, ivory slices were pretreated with CGMP for 1 hr. Thereafter, the CGMP-containing media were removed and the osteoclasts allowed to adhere to the mineral surface of the dentine.

CGMP and bovine serum albumin dissolved in alpha-MEM at a concentration of 10 mg/ml bound about 0.3 to 0.4 mM and about 0.2mM Ca, respectively, as measured with a calcium-electrode.

Results

The results are given in Table 1. First, the effect of CGMP on the formation of osteoclasts was assessed with the "Osteoclast Resorption Assay". CGMP was added at different concentrations to cells comprising a mixture of pre- and mature rat osteoclasts, osteoblasts that had been allowed to adhere to the surface of dentine slices (4.0x4.0x0.1)mm, prior to adding the test substance. Except where marked, all cultures were carried out in the presence of 10% fetal bovine serum (FBS). As control protein, bovine serum albumin was used. Cells were cultured for 24 hours. Thereafter the number of tartrate resistant acid phosphatase-positive multinucleated cells (TRAP+ MNC found on 16 individually cultured dentine slices, utilizing two different pools of osteoclasts (row 1). After scoring the osteoclasts, the same were removed by ultrasound. Thereafter the slices were washed, air-dried and mounted onto metal disks where they were sputter coated with gold. Using a light microscope equipped with a tangential light source, excavations were enumerated by two different persons.
The data shown the percentage of pits scored on 16 individual cultured dentine slices utilizing 2 different pools of osteoclasts (row 2). Row 3 depicts the ratio of pits and TRAP+MNC shown in rows 1 and 2.

Then, the effect of CGMP was investigated when the protein was added to the medium after the osteoclasts had attached to the mineral surface. As shown in Table1, row 1, CGMP had only a weak effect on the number of osteoclasts. The significant decrease at 10 mg/ml seems not to be specific since albumin had a similar effect. By contrast, the number of pits per ivory was decreased by CGMP and increased by albumin at a

concentration of 10 mg/ml (Table1, row 2). Calculating from row 1 and 2, the number of pits per osteoclast demonstrates that CGMP inhibits the bone resorbing activity of dissaggregated osteoclasts at a concentration of 1 and 10 mg/ml highly significantly (Table 1, row 3), whereas albumin stimulates this activity.

Table 1: The effect of CGMP on the formation of osteoclasts (row 1), on the resorptive activity of osteoclasts (row 2) and on the osteoclast resorption activity (row 3).

Effects of CGMP and Albumin on :	Albumin (mg/ml)				CGMP (mg/ml)				
	Ctrl	1.0	10.0	10.0 *	Ctrl	0.01	0.1	1.0	10.0
-the osteoclast recruitment (percent TRAP+MNC /dentine slice)	100	96	60	40	100	80	80	95	68
- the number of resorption pits (Percent Pits/ dentine slice)	100	90	180	107	100	72	64	55	35
- the osteoclast resorption activity (Ratio Pits / TRAP+MNC)	100	85	305	280	100	90	78	**58**	**54**

* without FBS

Example 3: Effects of CGMP on osteosarcoma cell viability/proliferation

The effect of CGMP on osteosarcoma cell (osteoblast-like cells) viability and proliferation was determined.

Materials and methods

1. Cells

HOS58 human osteosarcoma cells.

This cell line was characterized as a highly differentiated osteoblast-like osteosarcoma cell line (Kern et al., 1990, Calcif. Tiss. Int. **46**, Suppl.2, A54), which under appropriate conditions can be stimulated to a highly differentiated phenotype, as indicated by vitamin D receptors expression and mineralization.

TE 85 human osteosarcoma cells.

This cell line was obtained from ATCC (CRL1543), an osteogenic osteosarcoma cell line derived from a human 13y female. It can be grown to either high or low density,

depending on medium composition. These cells are rapidly growing osteoblast-like cells with characteristics of poorly differentiated early osteoblasts.

2. Colorimetric assay to quantify cell proliferation and viability with MTT-tetrazolium.

A modified method of T.Mosman's assay system (J. Immunol. Methods (1983) **65**, 55-63) was used to measure cell proliferation in growing cells and the viability of resting cells in growth-arrested cultures. At the end of treatment periods, either 48 or 72 hours, cells were incubated for 4 hours with 10 l MTT-tetrazolium stock solution (Sigma, 5 mg/ml PBS) per 100 l of cell culture medium in a CO_2 incubator at 37°C. After the reaction period, the medium-substrate mixture was carefully removed by suction and the dark-blue formazan crystals were dissolved overnight at 37°C in 100 l of 20% sodium dodecylsulfate (SDS). The optical density of the dye solution was measured at 550 nm.

Results

Effects of the CGMP on cell viability/proliferation was performed starting either from high cell density cultures, which had been first grown for 1 day at 10% FCS, followed by 2 days at 1% FCS and finally for 3 days in serum-free, BSA-supplemented medium with both agents or at lower initial cell density for 1 day with 10% FCS, followed immediately by treatment with the CGMP in serum-free, BSA-supplemented medium (table 2).

Table 2: The effect of CGMP on HOS 58 osteosarcoma cell (row 1), on TE 85 human osteosarcoma cell (row 2) MTT reaction.

	CGMP (mg/ml)					
	Ctrl	0.1	0.3	1	3	10
- Effects of CGMP on HOS 58 osteosarcoma cell MTT Reaction	0.16	0.18	0.19	0.3	0.33	0.37
- Effects of CGMP on TE 85 human osteosarcoma cell MTT Reaction	0.17	-	0.22	0.31	0.43	0.56

The results from both series of experiments showed, that the overall effects were similar at both culture conditions. CGMP induced a dose-dependent increase in MTT reactions with TE 85 cells being the most responsive cell type towards both agents. As shown in table 2, a dose of 10 mg/ml of CGMP maximally stimulated the metabolic activity of TE 85 by more than 3-fold. The same pattern was also observed in HOS 58 cells (Table 2).

Example 4 : Fermented milk containing CGMP

A traditional fermented milk with 1-4 % fats was prepared as follows:
After standardizing whole milk, low fat milk or a mixture of both, 0.05% by weight of CGMP as prepared in example 1 are added. The whole was pasteurized in a plate exchanger, the liquid was cooled to the fermentation temperature, a thermophilic or mesophilic lactic ferment was added and incubation was carried out until a pH of <5 was obtained.

Subsequent filling and sealing pots took place in a conventional manner.

Alternative embodiments having additions of 0.1 %, 0.25 % and 0.5% by weight of CGMPs have been prepared.

Example 5 : Fermented and gelled milk enriched in probiotic bacteria containing CGMP

Fermented and gelled milks were prepared enriched in probiotic bacteria. 89.3 parts milk containing fat were mixed with 3.7 parts of skimmed milk powder and about 0.05 by weight of CGMP as prepared in example 1, then the mixture was preheated to 70°C and pasteurized at 92°C/6 min, and after having been cooled to 43°C the mixture was inoculated with 2% of a common yogurt starter comprising Streptococcus thermophilus and Lactobacillus bulgaricus and with 5% of Lactobacillus johnsonii (La-1, CNCM I-1225). After conditioning in pots, fermentation was carried out at 38°C up to pH 4.6 and the pots were then cooled to 6°C.

The following amounts of CGMP were added: 0.1 %, 0.25 % and 0.5% by weight. Products thus obtained have been shown to have an increased stimulatory effect on the metabolic activity of bone forming cells and an inhibitory effect on bone resorption.

Example 6 : Fermented and gelled milk enriched in probiotic bacteria containing CGMP

Fermented and gelled milks are prepared as described in the previous example, wherein Lactobacillus johnsonii strain is replaced by Lactobacillus acidophilus La-10 (Nestlé Culture collection, Lausanne, Switzerland) (ATCC 11975).

Example 7 : Enteral composition containing CGMP

An enteral composition with an energy density of 6.3 kJ/ml and 8% (p/v) of proteins was prepared from "low temperature" skimmed milk powder, i.e. skimmed milk dried under controlled thermal conditions. 20 kg of the low temperature skimmed milk powder was dispersed in 100 kg of demineralized water at a temperature of about 50-55°C. This dispersion is microfiltered by passing demineralized water through until 600 kg of permeate have been eliminated. The retentate is then further concentrated to around 60 kg, which represents a dry matter content of 21% with a protein content, based on dry matter, of 82%.

To prepare the enteral composition, 2.3 kg of liquid retentate are mixed at 55°C with 600 g of maltodextrin, 200 g of sucrose, 20.3 g of Tri-K citrate H_2O, 9.2 g of $MgCl_26H_2O$, 5.8 g of NaCl and about 0.5 to 1 % by weight of CGMP as prepared in example 1. After the ingredients were dissolved in the retentate, demineralized water is added to a total weight of the dispersion of 4.7 kg. The pH was adjusted to 6.8, after which 300 g of fatty phase are introduced, the total weight of the dispersion being 5 kg.

After homogenization and sterilization, the product had an agreeable sugary taste. It has been shown to have an inhibitory effect on bone resoption and stimulatory effect on calcification.

Example 8 : Cereal bar containing CGMP

In order to prepare an expanded starting product, barley, wheat, corn or oat flour was treated in a twin-screw extruder for about 15 seconds at a screw speed of about 350 r.p.m. in the presence of approximately 12% of water. After the treatment, the expanded product left the extruder in the form of 2 to 3 mm long granules which were dried for 20 minutes at 100°C. The product thus obtained had a cellular structure and has the following composition:

Edible fibers	31%
Proteins	21%
Glucides	37.5%
Lipids	6.5%
Ash	2.4%
Water	1.6%

The expanded product was incorporated in a bar intended for treatment of diabetes, which had the following composition:

Expanded product	39.4%
Oat flakes	16.7%
Sorbitol	8.4%
Fructose	8.5%
Apple cubes	6.1%
Rice crispies	4.1%
Gelatine	4.0%
Abricot powder	2.5%
Palm oil	3.0%
CGMP	2.5% (as prepared in example 1)
Water	4.8%

Example 9 : Food supplement containing CGMP

A culture of the strain Lactobacillus johnsonii La-1 (CNCM I-1225) of human origin, was mixed with CGMP as prepared in example 1 and spray dried according to the process given in EP0818529 so as to obtain a food supplement containing an amount of about 5% by weight of CGMP.

The powder obtained may be used as a food supplement. A breakfast cereal, milk product or another food product may then be sprinkled with this powder containing CGMP.

Example 10 : Food supplement containing CGMP

A food supplement was prepared as described in example 9. However, Lactobacillus johnsonii was replaced by Lactobacillus acidophilus, La-10 (Nestec collection, Lausanne, Switzerland) or a mixture of the two strains.

Example 11 : Effect of CGMP on bone loss and calcium balance in a model of bone loss (tetracycline labelled rat)

A validated model of bone loss (the tetracycline labelled growing rat) was used to test whether CaCGMP and NaCGMP at 4.5 % of the diet reduced short-term bone loss and

influenced calcium absorption. It was shown that consumption of CaCGMP significantly reduced bone loss by approximately 20% compared to the control (p=0.01). NaCGMP reduced bone loss by 10% compared to the control (not significant). Furthermore, calcium absorption was higher in animals consuming either CaCGMP or NaCGMP, compared to the control group. Calcium retention rate was significantly higher in both the CaCGMP and NaCGMP groups compared to controls.

In the model used, both CaCGMP and NaCGMP positively influenced the absorption and retention of ingested Ca. Bone loss was significantly reduced by CaCGMP compared to the control, suggesting additional beneficial characteristics of the Ca form of CGMP over the Na form.

Effect of NaCGMP and CaCGMP on apparent Calcium absorption and retention

Mean values (with the standard deviation in parenthesis) for apparent Ca absorption (%), Ca retention (mg/day) and Ca retention rate (%) for the NaCGMP, CaCGMP and control groups are shown in Table 3 (below) and are graphically represented in Figure 1 . No apparent outliers appear, and the distribution of the data indicate that classic ANOVA methods for measuring differences between the groups are appropriate.

Table 3. Average values for apparent Ca absorption (%), retention (mg/day) and retention rate (%) (with the standard deviation in parenthesis) for the control, NaCGMP and CaCGMP groups.

Group:	**Ca Absorption (%)**	**Ca Retention (mg/day)**	**Ca Retention Rate (%)**
Control	63.00 (5.39)	26.13 (2.28)	61.52 (5.42)
CaCGMP	68.59 (5.25)	30.38 (2.52)	66.89 (5.53)
NaCGMP	68.83 (4.09)	28.11 (1.98)	66.67 (4.63)

Calcium absorption

Apparent Ca absorption for the CaCGMP and NaCGMP groups was, on average, 5.6% and 5.8% (respectively) greater than the average apparent Ca absorption for the control group. Statistical comparison of the three groups using Dunnett's test for multiple comparison showed that at least one group average was significantly different. However, using a confidence level of 95% the difference in Ca absorption between the two CGMP

groups and the control group was not statistically significant. No difference was found between the CaCGMP and NaCGMP groups.

Calcium retention

On average, the control group retained 26.1 (2.3) mg Ca/day, the CaCGMP group retained 30.4 (2.5) mg/day and the NaCGMP group retained 28.1 (2.0) mg/day, therefore, the estimated increase in Ca retention compared to the control was on average 4.25 mg/d for the CaCGMP group and 1.98 mg/d for the NaCGMP group. The Ca retention values for the two CGMP groups were compared to the control group using Dunnett's test for multiple comparison which showed that the CaCGMP group had significantly higher ($p<0.05$) retention of Ca compared to the control. The NaCGMP group showed a trend for higher retention of Ca, which was not statisitically significant. The two CGMP groups were compared using Tukey's multiple comparison test and no significant difference was found between the CaCGMP and NaCGMP groups.

Calcium retention rate

Ca retention rate was calculated in order to express the amount of Ca retained as a percentage of the amount of Ca consumed, thus taking into account the small differences in Ca intakes between the groups. Analysis of the Ca retention rate by one-way ANOVA gave a p-value of 0.088, meaning that at least one mean value could be significantly different. Using the Dunnett's test for multiple comparisons and a significance level of 10% it was demonstrated that CaCGMP and NaCGMP have a Ca retention rate which is on average roughly 5.2% (absolute) higher than the control, however these differences were both at the limit of significance.

Bone loss as quantified by Urinary [H^3] Tetracycline levels

[H^3] Tetracycline excretion was measured daily for 13 days in order to quantify bone loss. Group means are represented in figure 1. It can be clearly seen in figure 1 that excretion levels for [H^3] Tetracycline are approximately the same at the beginning of the experiment (Day 1) however the three groups appear to diverge from each other by day 13. Statistical comparison of the three groups using Dunnett's test for multiple comparison showed that at least one group average was significantly different ($p=0.01$). The groups were further compared using Tukey's multiple comparison test where the difference between CaCGMP and the Control was found to be statistically significant ($p<0.05$).

Remarkably, the quantity of CaCGMP consumed (approximately 660 mg/day) significantly reduced urinary tetracycline excretion and therefore bone loss, by

approximately 20%, compared to the control, in the animal model of bone loss used. Consumption of 720 mg of NaCGMP reduced tetracycline excretion by approximately 10% compared to the control, which was not significant.

The dry diets contained 4.5% of CaCGMP and 4.7% of NaCGMP and after mixing of the diets with approximately 40% water, the approximate daily consumption (calculated from the quantity of food consumed during the last 5 days of the experimental period) of CGMP was 660 mg of CaCGMP and 720 mg of NaCGMP. For an average sized adult this would be equivalent to approximately 18 g CGMP per day. If CaCGMP is ultimately targeted at the reduction of bone loss in postmenopausal women or the elderly (whose protein intakes can be suboptimal), a meal-replacer type product containing an upper limit of 18 g of CGMP would be the most likely application.

Example 12 : Effect of CGMP on bone loss and calcium balance in a model of menopausal bone loss (elderly ovariectomised rat)

Its was determined whether CGMP could reduce bone loss induced by estrogen deficiency in the ovariectomised rat over a three month period. Forty elderly rats were randomised to one of four groups Group A, B, C or D. Group A was SHAM operated (surgical procedure was carried out without ligation of the ovaries) and groups B, C and D were ovariectomised (i.e ligation of the ovaries). Group A and B were control groups and consumed a diet which was milk protein free. The diet of Group C contained 4.5% CaCGMP and that of Group D contained 4.5% NaCGMP. Baseline bone density measurements were taken 6 mm distal to the articular space prior to ovariectomy using quantitative computer tomography (pQCT). The measurement was repeated 5 weeks after ovariectomy and again 5 weeks later. As expected, there was no change in trabecular bone density (TBD) in Group A (SHAM control), Group B (ovariectomised control) lost approximately 16% TBD and surprisingly Group C (consuming CaCGMP) and Group D (consuming NaCGMP) lost 4.6% and 6.4% respectively, of their original TBD (Figure 2). This shows that CGMP plays an important role in the reduction of bone loss.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

Claims

1. A composition for prevention or treatment of a bone or dental disorder which comprises a milk protein hydrolysate.

2. A composition according to claim 1 wherein the milk protein hydrolysate is capable of inhibiting bone resorption or bone loss; or favouring calcium absorption, retention or calcification; or a combination thereof.

3. A composition according to claim 1 or 2 wherein the milk protein hydrolysate is caseinoglycomacropeptide (CGMP), a mimetic, homologue or fragment thereof which retains the ability of CGMP to inhibit bone resorption or bone loss; or favour calcium absorption, retention or calcification; or a combination thereof.

4. A composition according to claim 3 wherein the CGMP, mimetic, homologue or fragment thereof is in the form of a sodium or calcium salt.

5. A composition according to claim 3 or 4 which comprises an amount of about 0.01% to about 10% by weight dry matter of CGMP.

6. A composition according to any preceding claim which is in the form of a nutritional composition for a human or companion animal.

7. A composition according any preceding claim which contains a source of protein providing 7 to 25% of the total energy, a source of carbohydrate which provides 28 to 66% of the total energy, a source of lipids which provides 25 to 60% of the total energy, minerals and vitamins to meet daily requirements.

8. Use of a composition according to any preceding claim in the manufacture of a composition for the treatment or prevention of a bone or dental disorder.

9. A method of treatment or prevention of a bone or dental disorder which comprises administering an effective amount of a composition according to any one of claims 1 to 7.

Figure 1

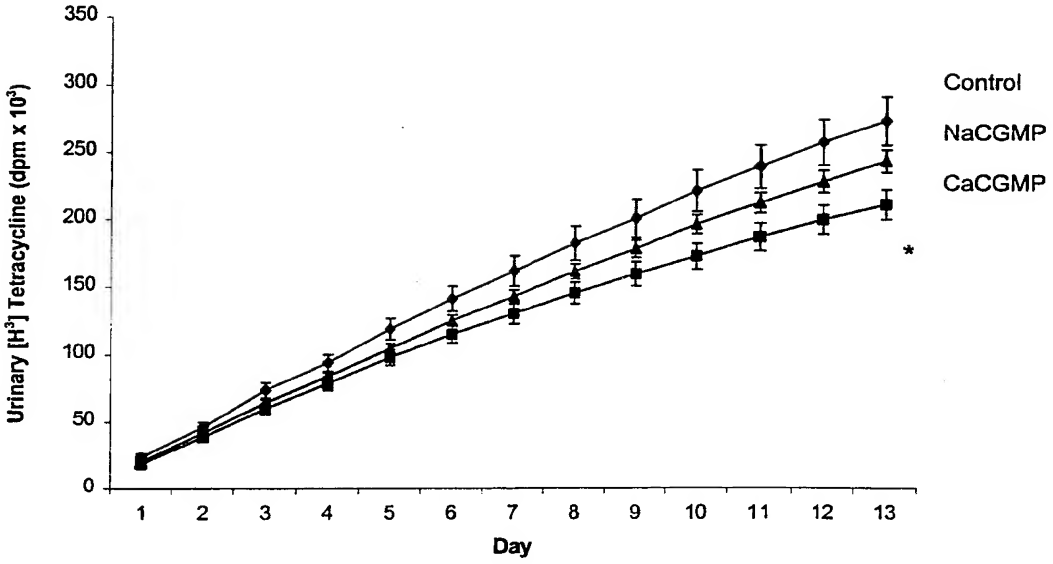
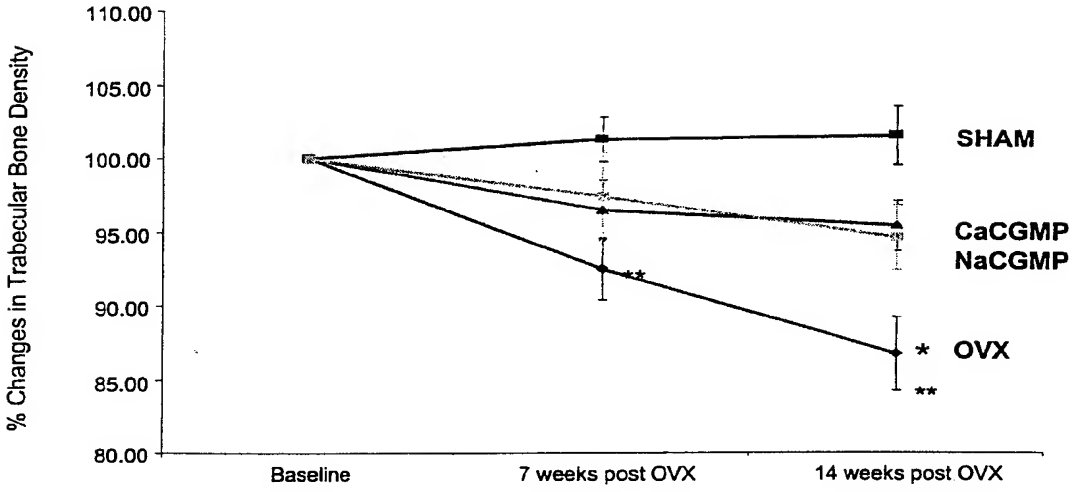

Urinary [H3] Tetracycline (dpm x 10³)
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Day
Control
NaCGMP
CaCGMP
*

Figure 2



§ *P*<0.05 for NaCGMP group compared to SHAM group at week 14

* *P*<0.05 for OVX control compared to CaCGMP and NaCGMP groups at week 14

** *P*<0.01 for OVX control compared to SHAM control at week 7 and week 14

INTERNATIONAL SEARCH REPORT

Int' tional Application No
PCT/EP 00/01562

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23J3/10 A23L1/305 A61K38/17 A61K38/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L A23J A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 5 670 201 A (DOUSAKO SHUN-ICHI ET AL) 23 September 1997 (1997-09-23) example 1	1-9

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2000	07.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bendl, E

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INTERNATIONAL SEARCH REPORT

Intr tional Application No
PCT/EP 00/01562

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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2

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/EP 00/01562

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 8 and 9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ,tional Application No
PCT/EP 00/01562

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